

METHOD FOR LOWERING BOTH SEQUENCE VARIATIONS' AND INCREASE OF
BASE LINE'S EFFECTS IN A DIAGNOSTIC HYBRIDISATION ASSAY, ASSAY
FOR PERFORMING SUCH A METHOD AND PROBE FOR USE IN THE ASSAY

5 The present invention relates to a method for
lowering the effect of sequences variations in diagnostic
hybridisation assays that use a nucleic acid probe to detect
an amplified nucleic acid analyte. The present invention also
relates to a method for lowering the unwanted IBL (Increase of
10 Base Line) effect, which limits the use of molecular beacons
in diagnostic hybridisation assays. The invention further
relates to the assays thus obtained and to probes for use in
such assays, the diagnostic assays and probes using at least
one of these two methods for lowering undesirable consequences
15 on diagnostic results. Many diagnostic assays are based on the
amplification of a nucleic acid molecule or part thereof with
the help of primers and the detection of the amplified
material by means of a probe. Under the appropriate reaction
conditions, the primers hybridise to the analyte to be
20 detected and initiate amplification of the target sequence.
This will lead to the generation of amplicons.

Meanwhile various amplification techniques have been
developed, such as PCR, LCR, NASBA, TMA, RCR, 3SR and SDA, and
are now well known to the person skilled in the art. Relevant
25 information on these techniques can be found in various
documents:

- PCR (Polymerase Chain Reaction), described in patents US-A-
4,683,195, US-A-4,683,202 et US-A-4,800,159, and its derivate
RT-PCR (Reverse Transcription PCR), to perform RNA
30 amplification disclosed particularly in EP-B-0.569.272,
- LCR (Ligase Chain Reaction), exposed in EP-A-0.201.184,

- NASBA (Nucleic Acid Sequence-Based Amplification) described in WO-A-91/02818, et
- TMA (Transcription Mediated Amplification) disclosed in patent US-A-5,399,491.
- 5 • RCR (Repair Chain Reaction) exposed in application WO-A-90/01069,
- 3SR (Self Sustained Sequence Replication) well explained in application WO-A-90/06995.

10 During or after amplification of the analyte or part thereof, the presence or amount of amplicons generated should be detected. This can be done with various known techniques such as separation of the sample on a gel with subsequent blotting and probing. This can only be done after the amplification is finished.

15 In a homogeneous procedure, amplification and detection occur without separating the reaction components. Amplicons are detected in the course of the amplification. Thus, the generation of amplicons can be monitored real-time and the data thus obtained can be used to determine the
20 presence or absence or the amount of the amplicon. One type of probe that is very useful in such homogeneous techniques is the molecular beacon.

Molecular beacons are single-stranded
oligonucleotides having a stem-loop structure. The loop
25 portion contains the sequence complementary to the target nucleic acid (either DNA or RNA). The stem is formed due to hybridisation of the complementary sequence of the 3' end with the 5' end. The stem can be unrelated to the target and is double-stranded. One arm of the stem is labelled with a
30 fluorescent dye (fluorophore), whereas the other one is coupled to a quenching molecule. In the stem-loop state the probe does not produce fluorescence because the energy of the

fluorophore is transferred to the quenching molecule. When the molecular beacon hybridises to the target the stem-loop structure is lost and the quencher and fluorophore are separated. At that stage the fluorescence emitted by the fluorophore can be detected and quantified. It was recently observed by the applicant during the development of assays using MB's for the detection of target, that the quality of commercially available enzymes (e.g. T7 RNA polymerase (T7)) that are normally used during the amplification process differs. This led to the observation that different batches of T7, even from the same supplier, with the same specific activity and volume activity, had different levels of unwanted opening of MB's (IBL effect) if these MB's consist of natural deoxyribonucleotides. Thus a fluorescent signal appears even in the absence of specific targeted sequences in the biological sample to be tested, generating possible false positive result.

If skilled people could have linked the IBL effect to T7 itself, our investigations showed unexpectedly that it is not the T7 itself that generates this phenomenon, but that it is more likely at least an unknown contaminant in the T7, which can differ from batch to batch. In line with the publications exposed in relation with the method for lowering the effect of sequences variations in diagnostic hybridisation assays, the man skilled in the art could have concluded to the usefulness of a MB that consists of only 2'-O-methyl derivatives to overcome the IBL effect. The applicant observed unexpectedly, that it is much more efficient to introduce less 2'-O-methyl groups in the MB, and that substituting all natural deoxyribonucleotides leads to non or less functional MB's.

Concerning the effect of sequences variations in diagnostic hybridisation assays, the amplicons generated in the amplification reaction can be detected quantitatively or qualitatively. In the former case the amount of amplicons generated is quantified. In a qualitative assay only the presence or absence of the analyte is determined.

Sequence variations (polymorphisms) in the analyte can lead to under-quantification thereof. Also in case of qualitative assays sequence variations can result in false negatives. It is generally assumed that this is caused by mismatches between the analyte and the primers used to amplify the analyte or by the structure of the analyte, which causes primers not to bind.

It is known that various polymorphic pathogenic strains of viruses exist as for example for the analytes HIV, CMV, HSV etc. These polymorphic strains differ from each other usually by one or more nucleotides. When a primer differs from the analyte it does not fit very well which leads to a reduced amplification. When the analyte is not linear but has a particular structure, it is less accessible by the primer, which in turn also leads to a reduced amplification.

In addition, sequence differences between the amplified target sequence of the analyte and the probe used for detection further lower the efficiency of detection. Analytes with polymorphisms, are thus less well detected than analytes that match perfectly with the consensus sequence of the probe. However, detection is usually performed at lower temperatures than the amplification, and therefore the negative effect of mismatches between probe and target is expected to be far less than is caused by differences between primer and analyte.

The probe can be optimised to fit known polymorphisms. However, in the case of unknown polymorphisms this is not possible. This is particularly a problem since new, unknown polymorphisms are continuously generated, which hamper a reliable detection or quantification especially in the case of HIV.

In the research that led to the invention it was now found that in a NASBA amplification reaction of a (clinical) sample of an HIV virus with an unknown polymorphism no signal or lower signals were generated in the Molecular Beacon detection system. However, surprisingly amplicons could be detected when the Molecular Beacon probe was modified such that the melting temperature of the probe with the analyte was increased. This led to the unexpected conclusion that amplicons were indeed produced but not or only partially detected.

Concerning the IBL effect, the applicant also observed that this effect, due to the unwanted opening of the stem of the molecular beacons by the action of contaminant(s) present within the enzymes' batch, such as the ones used during NASBA amplification (AMV-RT, T7 RNA polymerase and RNase H), can be lowered when the stem of the Molecular Beacon probes was modified in such a way that the nucleotides in the stem was partly replaced by 2'-O-Methyl derivatives.

Modification of the nucleotides constituting the probes and especially the Molecular Beacon probes is a widespread technique as can be derived from the publications mentioned below.

For example in application WO-A-00/66604, the use of L-ribo-LNA nucleotides in constructing oligonucleotides is exposed. L-ribo-LNAs can be used as a mean to increase affinity and/or specificity of the probes and also to equalize the affinity of different oligonucleotides for their complementary sequences. A special form of this LNA in combination with short probes could also be used to discriminate between RNA and DNA probes. This can be accomplished by replacing selected nucleosides in the oligonucleotide with L-ribo-LNAs to improve diagnostic and molecular biology procedures.

The publication of Majlessi et al. (vol. 26, no. 9, 1998, pages 2224-2229 (XP002241700)) describes linear probes containing only 2'-O-Methyl oligonucleotides. The goal of this publication is limited to a comparison between 2'-O-Methyl probes and normal 2'-deoxy probes, to show the improved T_m and the faster hybridization kinetics of the modified probes. They come to the conclusion that the variation of T_m values between matched and mismatched duplexes were much greater for 2'-O-Methyl probes than 2'-deoxy probes. This statement is true for all probes length examined (8-26 nucleotides) and the difference in T_m was most dramatic when probe lengths were 16 bases or less.

Another publication of Tsourkas et al. (vol. 30, no. 23, 1 December 2002, pages 5168-5174 (XP002241701)) proposes to decrease the nuclease degradation of Molecular Beacons. For this purpose, they protect the MB's and enhance the affinity between probes and RNA at the same time by replacing all 2'-deoxy nucleotides of a Molecular Beacon probe by 2'-O-Methyl oligonucleotides.

In a document of Brown-Driver et al. (vol. 9, no. 2, April 1999 (1999-04), pages 145-154 (XP009008881)), it is

proposed to use modified antisense oligonucleotides complementary to HCV RNA in place of unmodified oligonucleotides, to inhibit HCV translation. One of the modified oligonucleotide, which is a good candidate among
5 others, is the 2'-O-Methyl oligonucleotide. As set for the two above-mentioned publications, all the nucleotides of each modified antisense oligonucleotide are constituted by 2'-O-Methyl nucleotides. The consequence of this structure is the increase of the affinity and then the strong binding between
10 the target and the modified oligonucleotides.

In contrast to the here above mentioned prior art, the invention shows that Molecular Beacon probes incorporating just a few 2'-O-Methyl nucleotides per probe (between 6 and 15 of the hybridising 26 nucleotides) i.e. between 23% and 58% of
15 incorporation) are useful for RNA targets detection and even more efficient than oligonucleotides which are totally substituted with 2'-O-Methyl nucleotides.

Concerning the effect of sequences variations in
20 diagnostic hybridisation assays, LNA analogues are also good candidates to reach the final result of this invention: Molecular Beacons, which are partially modified with LNA's, become a very versatile tool for lowering the dependency towards sequence variation of the target in diagnostic assays.

25 In the same way, the IBL effect due to the possible opening of the stem-loop structure of the Molecular Beacons by contaminant(s) present in the amplification enzymes mixture is lowered when these Molecular Beacon probes are partially modified with 2'-O-Methyl nucleotides. This led to the
30 unexpected conclusion that with this improved structure the Molecular Beacon probes had a better stability and did not

open spontaneously in the presence of (contaminants of the amplification) enzymes.

It is therefore the object of the present invention
5 to lower the effects of sequence polymorphisms in an
hybridisation assay by manipulation of the affinity between
the probe and the analyte. This is achieved according to the
invention by introduction into the probe of one or more
nucleotides and/or nucleotide analogues that result in an
10 increase of the affinity between the analyte and the probe.

The invention thus relates to the use in a
diagnostic hybridisation assay of a probe, which could be a
Molecular Beacon, for lowering the effect of sequence
15 variations in a nucleic acid analyte, which assay comprises
the steps of contacting a set of primers and a sample
containing the nucleic acid analyte to amplify the analyte and
detecting the amplified analyte or its complement by means of
the probe, wherein the probe comprises one or more nucleotides
20 and/or nucleotide analogues that have an affinity increasing
modification and the diagnostic assay is for assessing the
amount of analyte present in the sample.

The invention also relates to the use in a
diagnostic hybridisation assay of a probe, which could be a
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variations in a nucleic acid analyte, which assay comprises
the steps of contacting a set of primers and a sample
containing the nucleic acid analyte to amplify the analyte and
detecting the amplified analyte or its complement by means of
30 the probe, wherein the probe comprises one or more nucleotides
and/or nucleotide analogues that have an affinity increasing
modification, i.e. at a constant temperature of hybridisation,

the melting temperature of the probe with any possible analyte's polymorphism is increased compared to the melting temperature of an unmodified probe with any analyte's polymorphism and the diagnostic assay is for assessing the presence of the analyte in the sample.

The invention concerns the use in a diagnostic hybridisation assay of a molecular beacon probe for lowering the IBL effect due to the possible opening of the stem of the molecular beacons by at least one contaminant present in the amplification enzymes mixture, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe's stem comprises:

- one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, especially 2'-O-methyl nucleotides, and
- one or more unmodified nucleotides.

The invention also concerns the use in a diagnostic hybridisation assay of a probe for lowering:

- the effect of sequence variations in a nucleic acid analyte, and/or
- the IBL effect due to the possible opening of the stem-loop structure of the molecular beacons by way of contaminated enzymes,

which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe's loop comprises:

- one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, and

- one or more unmodified nucleotides.

and/or the probe's stem comprises:

- 5 • one or more 2'-O-methyl nucleotides that have an affinity increasing modification, especially 2'-O-methyl nucleotides, and
- one or more unmodified nucleotides.

10 Concerning the lowering of the effect of sequence variations in a nucleic acid assay, the binding between a probe and a target is an equilibrium between unbound target and unbound probe on the one hand and the duplex between the two on the other.

15 This equilibrium is described by the melting temperature (T_m) of the duplex, which is defined herein as the temperature at which 50% of the probe is bound to the target nucleic acid in a duplex. Shifting the equilibrium towards the duplex can be achieved by an increase in the melting
20 temperature. In an assay this will lead to equal quantification of perfectly matching analytes, a better (i.e. higher) quantification of analytes containing polymorphisms in the sequence that is complementary to the probe and an improved detection of very small amounts of (polymorphic)
25 analytes.

As used herein the term "probe" is intended to comprise a stretch of nucleotides hybridising to the target. Preferably the hybridising part is a stretch of 10-50, more preferably 15-35, most preferably 15-30 nucleotides.

30 The term "affinity increasing" means that the melting temperature of a duplex between a probe comprising an

affinity increasing modification and an analyte is increased as compared to the melting temperature between the analyte and the unmodified probe.

5 The nucleotides or nucleotide analogues having a modification that increases the affinity of a probe containing them to DNA or RNA targets are preferably selected from the group consisting of 2'-O-derivatized nucleotides, locked nucleic acids (LNAs) and peptide nucleic acids (PNAs). In the case of 2'-O-derivatized nucleotides it is preferably a 2'-O-
10 methyl-nucleotide.

The probe according to the invention is preferably a so-called molecular beacon (MB). These probes recognize their targets with higher specificity than linear probes and can easily discriminate targets that differ from one another by a
15 single nucleotide. By the introduction of one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, and more particularly in the loop of the molecular beacon, the sensitivity to polymorphisms is lowered because the affinity of the probe to the polymorphic
20 analyte is increased and more accurate results are obtained in the assay. Thus, the modified molecular beacon becomes a very versatile tool for lowering the dependency towards sequence variation of the target in a homogenous assay.

With respect to the IBL effect, the term "Molecular Beacon" probe is intended to comprise a stretch of nucleotides belonging to the sequence of the 3' end of the probe hybridising with the complementary sequence of the probe's 5' end. Preferably the hybridising part is a stretch of 4-15, more preferably 5-10, most preferably 6-8 nucleotides.

The modified nucleotides can be used to synthesize the entire probe or to make a chimeric probe in which only a number of nucleotides is replaced by a modified nucleotide.

The amount of modifications that is minimally necessary to neutralize the mismatches is dependent on the amount of differences between the consensus sequence of an analyte and the sequence of the analyte to be detected. In general, it can be stated that the amount of modifications should be such that the melting temperature of the duplex between the probe and the analyte to be detected should lie above the detection temperature, i.e. the temperature at which the detection is performed.

The amount of modified nucleotides in a probe also depends on the type of modification used. The increase in T_m (the melting temperature of the probe with its target) upon introduction of a single LNA nucleotide in the probe is much higher as compared to the effect of a single 2'-O-methyl-nucleotide. In the Examples it is e.g. demonstrated that the introduction of two LNA nucleotides in the probe increased the T_m with 15°C, while twelve 2'-O-methyl-nucleotides were needed to obtain the same increase in T_m .

Analytes to be detected, such as HIV, may contain so-called "hot spots" that are prone to mutations and thus leading to polymorphisms. When the position of those hot spots or other positions of mismatches of the analyte and the probe are known, such as in the case of known isolates, it is

preferred to arrange the nucleotides or nucleotide analogues having an affinity increasing modification around the position of the polymorphism, preferably on conserved positions.

The present invention further relates to a
5 hybridisation assay using a nucleic acid probe to detect a nucleic acid analyte, wherein the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification. The modified nucleotides and/or analogues thereof used in the probe are as defined above.

10 The hybridisation assays of the invention may be of any kind in which a nucleic acid probe is used to detect a nucleic acid analyte. Such assays may be based on the detection of amplified analytes, such as in PCR-, TMA- or
15 NASBA-based assays. However, the probe can also be used in arrays. The invention can be used both in quantitative and qualitative diagnostic assays in which sequence polymorphisms of the target influence the reliability of the assay.

Diagnostic assays that benefit from the invention
20 are for example assays for detecting viruses, bacteria and other biomarkers as for example HIV, HBV, HCV, HSV, CMV, Ebola, *Legionella*, *Mycoplasma*, *Chlamydia*, *Bordetella*, RSV, MRSA, HSV, TNF- α , ER- α , as long as these diagnostic assays are characterised in such a way that they make use of the
25 hybridisation between the analyte of interest and the modified oligonucleotide.

The invention also relates to a probe that comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification. The probe is preferably a
30 molecular beacon.

In this application the terms [analyte], [amplicon],
and [target] or [target sequence] may be used interchangeably.

The analyte is the original nucleic acid molecule to be detected. The target sequence is the part of the analyte that is amplified by means of the primers. The amplification leads to formation of amplicons, which are the nucleic acid molecules that are physically detected by hybridisation to the probe. The sequence of the amplicons is the same or complementary to the target sequence within the analyte.

The invention will now be described in the examples and drawings that follow and which are not intended to limit the invention in any way. Then the invention will be further illustrated with reference to the accompanying drawings:

- Figure 1: Schematic representation of a Molecular Beacon allowing detection of *Legionella* bacteria, according to the state of the art, hereafter referred as *Legionella*.
- Figure 2: Schematic representation of an improved Molecular Beacon according to the present invention, that both allows detection of the same *Legionella* bacteria and lowers the IBL effect, hereafter referred as *Leg-met1*.
- Figure 3: Schematic representation of a Molecular Beacon allowing detection of *Mycoplasma* bacteria, according to the state of the art, hereafter referred as *Mycoplasma*.
- Figure 4: Schematic representation of a first embodiment of improved Molecular Beacon according to the present invention that both allows detection of the same *Mycoplasma* bacteria and lowers the IBL effect, hereafter referred as *Myco-met*.
- Figure 5: Schematic representation of a Molecular Beacon allowing detection of *Chlamydia* bacteria, according to the state of the art, hereafter referred as *Chlamydia*.
- Figure 6: Schematic representation of an improved Molecular Beacon according to the present invention that both allows detection of the same *Chlamydia* bacteria and lowers the IBL

effect, hereafter referred as Chlam-met.

- Figure 7: Schematic representation of a Molecular Beacon allowing detection of wild type of *Enterococci* bacteria, according to the state of the art, hereafter referred as WT Entero.
- Figure 8: Schematic representation of an improved Molecular Beacon according to the present invention that both allows detection of the same wild type of *Enterococci* bacteria and lowers the IBL effect, hereafter referred as WT Entero-met.
- Figure 9: Schematic representation of a Molecular Beacon allowing detection of HIV, according to the state of the art, incorporating 2'-O-methyl nucleotide neither in the stem nor in the loop, hereafter mentioned as Reference MB.
- Figures 10-19: Schematic representation of a series of Molecular Beacons allowing detection of HIV, according to the state of the art, hereafter referred as MB 1 to MB 10. Each Molecular Beacon has a different stem that contents 2'-O-methyl derivatives.
- Figure 20: Bar graph representing the effect of different T7 enzyme batches on the IBL effect.
- Figure 21: Bar graph representing the effect of optimal stem structure on the varying IBL effect of different enzyme batches, and representing the comparative study of IBL effect in relation with the structure of various Molecular Beacons used in an assay, i.e. unmodified Molecular Beacons on the one hand and modified Molecular Beacons on the other hand.

In figures 20 and 21, the modified Molecular Beacons incorporate 2'-O-methyl nucleotide in the stem.

In figures 1-8 and 10-19, the modified nucleotides are represented by the corresponding letter, mainly G for

guanidine or A for adenine, in bold, italic and underlined manner.

General description of experimental details of the examples:

5

The examples described below use the conditions and methods mentioned below, unless stated otherwise in the examples.

10 In the experiments that use amplified RNA material, this material was obtained by amplification of an RNA template (viral lysate), which was extracted and amplified using standard isolation and NASBA amplification conditions.

15 In every example the reaction conditions were kept identical except for the variation in the type of probe (Molecular Beacon) that was used.

As a model system to determine the improved subtype reactivity, a set of well-characterized viral lysates that represented most of the different subtypes of HIV-1 was used.

20 If an amplification reaction is being used to generate a quantitative result, the target has been co-amplified using a well-known amount of internal calibrator, which has been added before the isolation. This internal calibrator is detected using another Molecular Beacon with a
25 different colour. From the real-time amplification curves and using specially developed software it is possible to accurately determine the amount of material that is present. Melting temperatures (T_m 's) between target and Molecular Beacons were determined by measuring the fluorescence
30 intensity of the mixtures as a function of the increasing temperature. From these melting curves the T_m can be determined. If the excess target is amplicon (RNA) the T_m is

referred to as T_m loop RNA. If the target is an excess of synthetic DNA the T_m is referred to as T_m loop DNA. The latter has been determined using synthetic DNA strand, which is complementary towards the Molecular Beacon. In this sequence
 5 we have introduced two C's opposite to the Inosines. In the RNA target (amplicon) these positions contain two T's.

EXAMPLE 1

10 Effect of the replacing nucleotides in a probe with 2'-O-methyl derivatives on the T_m of the duplex

The sequences of the hybridising part of three new and one reference MB are depicted in Table 1. Also the T_m 's values of the different Molecular Beacons that have been
 15 measured for synthetic DNA and for amplified material (RNA) are shown in Table 1.

Table 1

Name	T_m	T_m	Position																									
	loop DNA	loop RNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref.	58.6	48.6	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Me-1	54.7	53.8	A	T	C	<u>A</u>	<u>A</u>	T	G	<u>A</u>	G	G	<u>A</u>	I	G	C	T	G	C	<u>A</u>	G	<u>A</u>	I	T	G	G	G	A
Me-2	58.7	62.1	A	T	C	<u>A</u>	<u>A</u>	T	<u>G</u>	A	<u>G</u>	<u>G</u>	<u>A</u>	I	<u>G</u>	C	T	<u>G</u>	C	A	<u>G</u>	<u>A</u>	I	T	<u>G</u>	<u>G</u>	G	A
Me-7	59.4	> 65	A	T	C	<u>A</u>	<u>A</u>	T	<u>G</u>	A	<u>G</u>	<u>G</u>	<u>A</u>	I	<u>G</u>	C	T	<u>G</u>	C	<u>A</u>	<u>G</u>	<u>A</u>	I	T	<u>G</u>	<u>G</u>	G	A

20 A, G = 2'-O-Me nucleotides of A and G respectively

As can be seen from the data in Table 1, the introduction of 2'-O-methyl derivatives leads to an increased affinity (higher T_m loop RNA) between target RNA and the
 25 Molecular Beacon's loop. As expected, this increase is less

pronounced for the DNA-Molecular Beacon complex, since 2'-O-methyl derivatives are known to bind stronger with RNA than DNA targets.

5

EXAMPLE 2**Effect of replacing nucleotides in a probe with LNAs on the T_m of the duplex**

In Table 2, the hybridizing sequences of two new MB's are shown together with the reference MB. The new MB's contain respectively two and three LNA building blocks. Also the T_m values for complementary DNA and for amplified material (RNA) has been measured and results are depicted in the table. From this Table it can be seen that LNA nucleotides increase the T_m of both DNA and RNA complexes. Also it can be seen that the effect on the T_m per modification is much higher as compared to the 2'-O-methyl nucleotides.

Table 2

Name	T_m	T_m	Position																									
	loop DNA	loop RNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref2	45.8	57.6	A	T	C	A	A	T	G	A	G	G	A	A	G	C	T	G	C	A	G	A	A	T	G	G	G	A
LNA1	47.1	62.8	<u>a</u>	T	C	A	A	T	G	A	G	G	A	A	G	C	<u>t</u>	G	C	A	G	A	A	T	G	G	G	A
LNA2	57.5	> 65	<u>a</u>	T	C	A	A	T	G	A	G	G	<u>a</u>	A	G	C	<u>t</u>	G	C	A	G	A	A	T	G	G	G	A

20

a LNA nucleotide

EXAMPLE 3

Detection of different HIV-1 isolates with the modified Molecular Beacons

25

The HIV-1 isolates which have been selected to act as a model system to investigate the effect of five sequence polymorphisms in the target RNA sequence are shown in Table 3. These materials were available as viral lysates with a known concentration and have been used in a NASBA amplification with the Molecular Beacon's of example 1 and 2.

Table 3

Name	Position																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Ref. Molecular Beacon	A	T	C	A	A	T	G	A	G	G	A	I	G	C	T	G	C	A	G	A	I	T	G	G	G	A
Subtype A	A	T	C	A	A	T	G	A	G	G	A	<u>A</u>	G	C	T	G	C	A	G	A	<u>A</u>	T	G	G	G	A
Subtype G1	A	T	<u>T</u>	A	A	T	G	A	<u>A</u>	G	A	<u>A</u>	G	C	T	G	C	A	G	A	<u>G</u>	T	G	G	G	A
Subtype N	A	T	C	A	A	T	G	A	G	G	A	<u>A</u>	G	C	<u>A</u>	G	C	A	G	A	<u>C</u>	T	G	G	G	A
Subtype O1	A	T	C	A	A	T	G	A	<u>T</u>	G	A	<u>A</u>	G	C	<u>A</u>	G	C	A	G	A	<u>T</u>	T	G	G	G	A
Subtype O3	A	T	C	A	A	T	G	A	G	G	A	<u>A</u>	G	C	<u>G</u>	G	C	A	G	A	<u>T</u>	T	G	G	G	A

The quantification results that were obtained for samples containing an equal amount of target per subtype with three of these Molecular Beacons as compared to our reference Molecular Beacon are shown in Table 4.

Table 4

subtype	Ref MB	LNA2	Me-2	Me-7
A	6.5	6.5	6.5	6.5
G	4.7	5.9	6.4	6.6
N	4.3	4.6	5.2	5.3
O1	3.3	4.9	5.4	5.9

O3	4.9	5.8	6.3	6.2
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Since the A-subtype fits perfectly with all four Molecular Beacon's, all quantification data are normalized based on this subtype. The other subtypes have been selected since they show sequence variation with the loop-binding region of the Molecular Beacon (see Table 3). As can be seen, the highest quantifications for all five subtypes have been observed with the Me-7 Molecular Beacon derivative.

To investigate the relationship between the better quantification and the T_m loop RNA value ($^{\circ}\text{C}$) of the different Molecular Beacon's, the latter were determined for the duplex between the Molecular Beacon derivatives and the NASBA amplicons. This yielded the results shown in Table 5.

Table 5: T_m loop RNA values ($^{\circ}\text{C}$) for amplified material (RNA) with several MB's

subtype	ref MB	LNA2	Me-2	Me-7
A	48	65	62	65
G	45	52	58	65
N	42	55	53	60
O1	27	44	44	53
O3	40	52	54	60

As can be seen from Tables 4 and 5 a clear correlation can be observed between a higher T_m loop RNA (between the amplicon and the Molecular Beacon) and a higher quantification.

Example 4

Effect of the Stem structure on the IBL reduction

A series of MB's were compared that had an identical hybridising sequence towards an HIV target. The difference between the MB's was the sequence of the stem, and the content and place of 2'-O-methyl derivatives in the stem. The investigated structures are depicted in Figures 10-19. Figure 9 shows the Molecular Beacon that is used as the reference and does not contain 2'-O-methyl derivatives.

All MB's in the experiment described below were supplied to standard NASBA amplification conditions in the absence of target that could be detected with these MB's (no template reactions). The signal of the fluorophore present in the MB was measured as a function of time. From these data the IBL effect is obtained as the increase of the signal over a time period of 60 minutes. The percentage IBL is the increase as compared to the starting signal.

Table 6

MB number	IBL percentage
Reference MB	7 %
MB 1	6 %
MB 2	5 %
MB 3	3 %
MB 4	9 %
MB 5	5 %
MB 6	4 %
MB 7	5 %
MB 8	1.5 %
MB 9	0.5 %
MB 10	10 %

From this table it can be concluded that MB 4 and MB 10, which contain continuous stretches of 2'-O-methoxy nucleotides at either the 3' or the 5' end of the MB do not work optimally. It can also be seen from these data that the design of a stem with a low IBL effect was unexpectedly low for MB 8 and MB 9.

Example 5

Effect of different T7 enzyme batches on the IBL effect

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A MB that was designed for Enterovirus (figure 7) was compared with a MB that contained the same hybridizing sequence, but the stem structure was based on the MB 8 (see Figure 17) from the previous example. The modified MB that was designed for Enterovirus is depicted in Figure 8.

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The two MB's were studied in the experiment described below. They were supplied to standard NASBA amplification conditions in the absence of target that could be detected with these MB's (no template reactions). Reaction conditions were kept identical for both MB's and the variations that were studied was:

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- 1) the effect of the introduction of the new stem in the probe and
- 2) the effect of three batches of the T7 enzyme in the Nasba reaction.

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The three batches were obtained from the same vendor and with the same specific activity. They were different in the observed IBL effect (normal, intermediate, bad in terms of IBL effect (bad being a large amount of unwanted opening of the MB)). The signal of the fluorophore present in the MB was measured as a function of time. From these data the IBL effect

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is obtained as the increase of the signal over a time period of 120 minutes. The percentage IBL is the increase as compared to the starting signal.

From Figure 20, it can be seen that for all MB's containing normal deoxynucleotides, the so-called "WT Entero", the unwanted opening of the Molecular Beacons produces a dramatic increase of the IBL effect arising between the use of intermediate and the use of bad T7's batches. However, the modified MB's containing 2'-O-methoxy's in the stem (figure 8), show hardly any influence on the quality of the T7 batches. This clearly shows that it is the quality of the T7 (e.g. at least one contaminant of the T7 could be present) that determines the IBL and not the T7 itself.

Example 6

Effect of optimal stem structure on the varying IBL effect of different enzyme batches

A second series of MB's that were designed for different targets (*Legionella* (Figure 1), *Chlamydia* (Figure 3), *Mycoplasma* (Figure 5)) were compared with MB's that contained the same hybridising sequence, but the stem structure was based on the MB 8 (see Figure 17) from the previous example (respectively *Legionella* (Figure 2) and *Chlamydia* (Figure 6)). For the *Mycoplasma* target this was not possible, so a small deviation on the stem structure was made as compared to the MB 8 and modified Molecular Beacons specific for this target were obtained (see Figure 4).

MB's were studied in pairs (per target) in the experiment described below. They were supplied to standard NASBA amplification conditions in the absence of target that

could be detected with these MB's (no template reactions). Reaction conditions were kept identical per set of MB's and the variation that was studied was the introduction of the new stem in the probe and the second variation was the use of two
5 batches of the T7 enzyme in the Nasba reaction. The first batch (referred to as T7) was known to be good, and the second batch (from the same vendor and with the same specific activity) was known to generate more unwanted opening of the Molecular Beacons producing a dramatic increase of the IBL
10 effect. The signal of the fluorophore present in the MB was measured as a function of time. From these data, the IBL effect is obtained as the increase of the signal over a time period of 60 minutes. The percentage IBL is the increase as compared to the starting signal.

15 From Figure 21, it can be seen that for all MB's containing normal deoxynucleotides, the bad T7 results in a higher effect of the unwanted IBL. However, the modified MB's containing 2'-O-methoxy's in the stem showed hardly any influence on the quality of the T7. In other words, the use of
20 methoxy derivatives in the stem structure makes the MB less vulnerable to the enzyme quality.